

**Tat-derived Oligourea and Its Method of Production
and Use in High Affinity and Specific Binding
of HIV-1 TAR RNA**

This work was supported in part by the National Institutes of Health Grants Al 34785 and Al 01369, TW 00702. Tariq M. Rana is a recipient of Research Career Development Award from NIH.

5

Field of the Invention

This invention relates to a synthesized oligourea containing the basic-arginine rich region of Tat, the method of production of this oligourea and the use thereof. In particular, this invention relates to the design of drugs comprising the oligourea backbone of the invention, further comprising amino acid side chains. Similarly, the DNA-binding oligourea of the invention can also be synthesized to control biological processes involving DNA-protein interactions

Background of the Invention

Various scientific and scholarly articles are referred to in brackets and footnotes throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains. Full citations of the references appear at the end of the specification.

Protein-nucleic acid interactions are involved in many cellular functions such as transcription, RNA splicing, and translation. Small peptides with unnatural backbones that can bind with high affinity to a specific sequence or structure of nucleic acids and interfere with protein-nucleic acid interactions would provide useful tools in molecular biology and medicine. Recently, minor-groove-binding polyamide ligands have been designed for sequence-specific recognition of DNA.¹ In contrast to DNA, RNA molecules can fold into extensive structures

-2-

containing regions of double-stranded duplex, hairpins, internal loops, bulged bases and pseudo-knotted structures.² The complexity of RNA structure makes it difficult to design ligands for sequence-specific RNA-recognition. Three-dimensional structures of RNA create binding sites for specific interactions with proteins.

One example of such interactions is the mechanism of trans-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the trans-activation responsive region (TAR) RNA, a 59-base stem-loop structure located at the 5'-end of all nascent HIV-1 transcripts.³ Replication of human immunodeficiency virus type 1 (HIV-1) requires specific interactions of Tat protein with the TAR RNA. Inhibition of Tat-TAR interactions is a potential approach for anti-HIV therapeutics. Since structural information is now available for TAR RNA and TAR-Tat peptide complexes from NMR⁴, photocrosslinking,⁵ and affinity cleaving studies,⁶ it is possible to design small molecules to interfere with Tat-TAR function. We have recently begun to examine TAR RNA recognition by unnatural biopolymers.⁷

Objects of the Invention

It is an object of the invention to provide substances which have higher binding affinities for RNA than natural peptides, which are resistant to proteases and which can interact with nucleic acids in a fashion similar to natural peptides. Such substances can be used to inhibit protein-nucleic acid interactions important for cellular processes.

It is a further object of the invention to provide a substance inhibiting protein-nucleic acid interactions. In particular, it is an object of the invention to provide a substance which controls

-3-

biological processes involving DNA-protein interactions, and which inhibit transcription in HIV-1 infected cells. Such a substance leads to the design of drugs based on the substance.

5 The current invention comprises a novel synthesized oligourea containing the basic-arginine rich region of Tat. The oligourea of the invention shows specific recognition of HIV-1 TAR RNA.

10 Other objects and advantages of the invention will become apparent to those skilled in the art from the accompanying description of the invention.

Brief Description of the Drawings

15 **Figure 1A:** The Tat-derived peptide, amino acids 48 to 57, contains the RNA-binding domain of Tat protein (SEQ ID NO:1). **Figure 1B:** Structure of the generic oligourea backbone. Side-chains corresponding to a desired amino acid are substituted at the R₁ and R₂ positions. Sequence of Tat-derived oligourea corresponds to the side-chains of the Tat peptide shown in (A), except the addition of an L-Tyr amino acid at the carboxyl-terminus. Tat-derived oligourea was synthesized on solid support by using activated *p*-nitrophenyl carbamates and azides of protected amines followed by
20 reduction with SnCl₂-thiophenol-triethylamine (Kim, J. M.; Bi, Y. Z.; Paikoff, S. J.; Schultz, P. G. *Tetrahedron Lett.* 1996, 37, 5305- 5308; Kick, B.; Bllman, J. *J Med. Chem.* 1995, 38, 1427-1430; incorporated by reference herein). After cleavage from the resin, the oligourea
25 was purified by HPLC on a Zorbax 300 SB-C₈ column (Wang, Z.; Rana, T. M. *J Am. Chem. Soc.* 1995, 117, 5438-5444; Wang, Z.; Rana, T. M. *Biochemistry* 1996, 35, 6491-6499; Wang, Z.; Wang, x.; Rana, T. M. *J Biol. Chem.* 1996, 27, 16995-16998; incorporated by reference herein). The mass

30

-4-

of fully deprotected and purified oligourea was confirmed by ES and MALDI mass spectrometry; 1849.2 (M +H).

Figure 2A: Secondary structure of wild-type TAR RNA used in this study. Wild-type TAR RNA spans the minimal sequences that are required for Tat responsiveness *in vivo*¹⁴ and for *in vitro* binding of Tat-derived peptides.⁹ Wild-type TAR contains two non-wild-type base pairs to increase transcription by T7 RNA polymerase. Mutant M0 TAR contained no bulge residue in its sequence. In mutant G26C, a base-pair in the upper stem of TAR RNA, G26-C39 was substituted by C26-G39.

Figure 2B: Electrophoretic mobility shift analysis for the Tat-derived oligourea binding to wild-type and trinucleotide bulge mutant (M0) TAR RNA. 5'-end labeled TAR RNAs (40 nM) were heated to 85 °C for three minutes and then cooled to room temperature in TK buffer (50 mM Tris-HCl pH 7.4), 20 mM KCl, 0.1% Triton X-100). The oligourea (150 nM) was added to wild-type or mutant TAR and incubated at room temperature for one hour. After adding 30% glycerol, the oligourea-RNA complexes were resolved on a non-denaturing 12% acrylamide gel and visualized by autoradiography or phosphorimaging. **Figure 2C:** Specificity of the oligourea-TAR complex formation determined by competition experiments. Oligourea-RNA complexes were formed in the presence of increasing concentrations of unlabeled wild-type or mutant TAR RNAs. Concentrations of the competitor RNAs in lanes 3, 4, 5, 6 were 50, 100, 150, and 200 nM, respectively. Lanes 1 and 2 were marker lanes showing RNA and oligourea-RNA complexes. Oligourea-RNA complexes are labeled as R-P.

Figure 3. Site-specific photocrosslinking reaction of TAR RNA labeled with 4-thioUracil at position 23 with the oligourea. For photochemical reactions, RNA

-5-

duplex was prepared by hybridizing two strands.^{5,7} Strand 1 of the duplex was 5'-end labeled with 32p Preformed RNA duplexes (40 nM) in the absence or presence of the oligourea (100 riM) were irradiated (360 um) and analyzed by denaturing gels as described earlier.^{5,7} Proteinase K digestion was performed at 55 °C for fifteen minutes after LW irradiation. R-R and R-P XL indicate the RNA-RNA and RNA-oligourea crosslink, respectively.

Figure 4. Inhibition of Tat transactivation by the oligourea derivative *in vivo*. CAT activity expressed from the integrated HIV-1 LTR of HL3TI Cells with increasing amounts of oligourea is shown. Luciferase activity was a control experiment to monitor the transfection inhibition of pSV2Tat by the addition of oligourea. CAT and Luciferase activities were measured from multiple experiments and normalized to 100%. Control lane (labeled as positive) shows Tat transactivation in the absence of oligourea.

20

Detailed Description of the Invention

The invention provides a composition and method for inhibiting the interaction of a nucleic acid and specific binding protein *in vitro* and *in vivo*. The composition is an oligourea backbone as disclosed in figure 1B, with amino acid side-chains substituted at the R₁ and R₂ positions. In accordance with the invention, it has been discovered that the rigid and protease insensitive oligourea backbone, when substituted with a sequence of amino acid side-chains modeled after a known nucleic acid binding domain, will mimic the nucleic acid binding domain in specificity, but with a much lower disassociation constant. This nucleic acid binding composition may be used for research into the physiological effects of nucleic acid binding proteins,

-6-

assay methods for detecting nucleic acids and therapeutic methods for inhibiting protein-nucleic acid interactions that lead to disease states. Also provided, is a method for inhibiting protein-nucleic acid interactions *in vitro* and *in vivo* which entails introducing the oligourea molecules of the invention.

The composition of the invention is composed oligourea backbone, the generic form of which is disclosed in Fig. 1B, which supports the side chains of amino acids, at the R₁ and R₂ position of Fig. 2B. When the oligourea molecule has amino acid side chains that correspond to the side chains of a nucleic acid binding protein in composition and sequence, the oligourea molecule then binds to the target nucleic acid specifically and with a very low disassociation constant. In a preferred embodiment, the oligourea molecule has a dissociation constant upon binding the target nucleic acid of less than or equal to 0.7 μ M (less than or equal to 0.5 μ M more preferred; less than or equal to 0.3 μ M most preferred). In Example 1, the use of an oligourea molecule of the invention is illustrated which mimics the RNA-binding protein Tat. In a preferred embodiment, the oligourea molecule is comprised of amino acid side chains that mimic the Tat molecule. In a more preferred embodiment, the side-chains correspond to residues 48 - 57 of the Tat molecule, more preferred, SEQ ID NO:1. In a most preferred embodiment, the amino acid side-chains correspond to SEQ ID NO:1 with a L-Tyr amino acid at the carboxyl-terminus.

The composition of the invention encompasses a very diverse assortment of molecules, all with oligourea backbones and amino acid side-chains. The oligourea molecule may be any length which achieves the desired dissociation constant from the nucleic acid. In a preferred embodiment, the oligourea is 3 to 50 urea-units

-7-

long (5 to 30 more preferred, 8 to 25 most preferred). The oligoureia molecule may comprise amino acid side-chains that correspond to the binding region of any nucleic acid binding protein presently known or that will be discovered. Types of DNA binding proteins of interest include, but are not limited to, transcription control proteins (e.g. transcription factors, see Conaway and Conaway, 1994, Transcription Mechanisms and Regulation, Raven Press Series on Molecular and Cellular Biology, Vol. 3, Raven Press, Ltd., New York, NY), recombination enzymes (e.g. *hin* recombinase), DNA modifying enzymes (e.g. restriction enzymes), structural proteins (e.g. histones and nonhistone chromatin proteins such as HMG proteins), single-stranded DNA-binding proteins (e.g. those involved in the propagation of a DNA replication fork or in the packaging of T-DNA ssDNA) and double- and single-stranded RNA-binding proteins. RNA-binding proteins are also contemplated in regard to the present invention, (see, for example, Draper DE, J Mol Biol 1999 Oct 22;293(2):255-70; Haile DJ, Am J Med Sci 1999 Oct;318(4):230-40; Cusack S, Curr Opin Struct Biol 1999 Feb;9(1):66-73).

Transcription factors suitable for use with the present invention include, but are not limited to, homeobox proteins, zinc finger proteins, hormone receptors, helix-turn-helix proteins, helix-loop-helix proteins, basic-Zip proteins (bZip) and β -ribbon factors (see Harrison, 1991, Nature 353:715-719). Homeobox DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, HOX, STF-1 (Leonard et al., 1993, Mol. Endo., 7:1275-1283; Scott et al. (1989), Biochem. Biophys. Acta, 989:25-48), Antp, Mat α -2 and INV. Zinc finger DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, Zif268, GLI and XFin. For

-8-

reviews of zinc-finger DNA-binding proteins see Klug and Rhodes (1987), Trends Biochem. Sci., 12:464; Jacobs and Michaels (1990), New Biol., 2:583; and Jacobs (1992), EMBO J., 11:4507-4517. Hormone receptor DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, glucocorticoid receptor, thyroid hormone receptor and estrogen receptor (see, e.g., U.S. Pat. Nos. 4,981,784; 5,171,671; and 5,071,773). Helix-turn-helix DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, λ -repressor, cro-repressor, 434 repressor and 434-cro (See, e.g., Pabo and Sauer, 1984, Annu. Rev. Biochem., 53:293-321). Helix-loop-helix DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, MRF4 (Block et al., 1992, Mol. and Cell Biol., 12(6):2484-2492), CTF4 (Tsay et al., 1992, NAR, 20(10):2624), NSCL, PAL2 and USF (see, for review, Wright (1992), Current Opinion in Genetics and Development, 2(2):243-248; Kadesch, T. (1992), Immun. Today, 13(1):31-36; and Garell and Campuzano (1991), Bioessays, 13(10):493-498). Basic Zip DNA-binding proteins contemplated for use with the instant invention include, but are not limited to, GCN4, fos and jun (see, for review, Lamb and McKnight, 1991, Trends Biochem. Sci., 16:417-422). β -ribbon factors contemplated for use with the instant invention include, but are not limited to, Met-J, ARC, and MNT.

The oligourea composition of the invention has a diverse range of uses. Any application that requires a strong nucleic acid binding molecule may use the oligourea molecules of the invention. The oligourea molecules may be used to inhibit the native nucleic acid binding molecule by competing with the native protein molecule for the binding site on the nucleic acid. This

-9-

application may be used for research purposes or for therapy purposes. In therapeutic methods, the oligourea molecules of the invention may be used to inhibit a protein-nucleic interaction that leads to a disease state. Example 1 illustrates the use of an oligourea molecule to inhibit the interaction between the Tat protein and the TAR RNA from HIV. Finally, the oligourea molecule may be used to detect the presence of the target nucleic acid molecule in any method that requires the detection and/or quantization of a specific nucleic acid.

A method to inhibit the interaction between a specific interaction between a binding protein and its target nucleic acid comprising introducing an oligourea molecule that specifically competes with the binding protein for the binding site on the target nucleic acid. In a preferred embodiment, the method is a therapeutic method for patients in need of such a treatment. This method is particularly suited as therapeutic method because of the high specificity of the inhibition provided. The therapeutic method is applicable to any disease state in which a nucleic acid-protein interaction affects the disease state. In a preferred embodiment, the patient is human. In a more preferred the patient is infected by the HIV-1 virus, and the oligourea molecule introduced comprises amino acid side chains that correspond to the Tat molecule.

The following Example sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular

-10-

Biology, John Wiley & Sons (2000) (hereinafter "Ausubel et al.") are used.

The following example is provided to describe the invention in greater detail. It is intended to
5 illustrate, not to limit, the invention.

Example I

We synthesized an oligourea containing the
10 basic-arginine rich region of Tat by solid phase synthesis methods, and tested for TAR RNA binding. This tat-derived unnatural biopolymer binds TAR RNA specifically with affinities higher than the wild-type Tat peptide. Site-specific photocrosslinking experiments
15 using a photoactive analog (4-thio-uracil) containing TAR RNA revealed that the unnatural biopolymer interacts with RNA in the major groove. The oligourea-RNA complexes were stable to proteolytic digestion. RNA recognition by an oligourea provides a new class of RNA-binding
20 molecules that can be used to control cellular processes involving RNA-protein interactions in vivo.

In this report, we synthesized an oligourea containing the basic-arginine rich region of Tat by solid phase synthesis methods, and tested for TAR RNA binding.
25 Oligoureas have backbones with hydrogen bonding groups, chiral centers, and a significant degree of conformational restriction. Introducing additional side chains at the backbone NH sites can further modify biological and physical properties of these oligomers.
30 This tat-derived unnatural biopolymer binds specifically to TAR RNA with affinities higher than the wild-type Tat peptide. These results identify a new class of unnatural peptides for structure-specific recognition of RNA.

The promoter of HIV-1, located in the U3 region
35 of the viral long terminal repeat (LTR), is an inducible

-11-

promoter which can be stimulated by the trans-activator protein, Tat.³ As in other lentiviruses, Tat protein is essential for trans-activation of viral gene expression.⁸ A number of studies showed that Tat-derived peptides which contain the basic arginine -rich region of Tat are able to form *in vitro* complexes with TAR RNA.⁹ We synthesized a tat-derived oligourea containing the basic-arginine rich region of Tat protein by solid phase synthesis methods (Figure 1). Recently, two methods have been reported for solid phase synthesis of oligourea.^{10, 11} To synthesize Tat-derived oligourea on solid support, we used activated *p*-nitrophenyl carbamates and protected amines in the form of azides, which were reduced with SnCl₂-thiophenol-triethylamine on solid support.^{11, 12} After HPLC purification and characterization by mass spectrometry, the oligourea was tested for TAR RNA binding (Figure 2). The tat-derived oligourea was able to bind TAR RNA and failed to bind a mutant TAR RNA without the bulge residues.

Equilibrium dissociation constants of the oligourea-TAR RNA complexes were measured using direct and competition electrophoretic mobility assays.¹³ Dissociation constants were calculated from multiple sets of experiments which showed that the oligourea binds TAR RNA with a K_D of $0.11 \pm 0.07 \mu\text{M}$. To compare the RNA-binding affinities of the oligourea to natural peptide, we synthesized a tat-derived peptide (Tyr47 to Arg57) containing the RNA-binding domain of Tat protein (Figure 1). Dissociation constants of the Tat peptide-RNA complexes were determined from multiple sets of experiments under the same conditions used for oligourea-TAR RNA complexes. These experiments showed that the Tat peptide (47-57) binds TAR RNA with a K_D of $0.78 \pm 0.05 \mu\text{M}$. A relative dissociation constant (K_{REL}) can be determined by measuring the ratios of wild-type Tat peptide to the

-12-

oligourea dissociation constants (K_D) for TAR RNA. Our results demonstrate that the calculated value for K_{REL} was 7.09, indicating that the urea backbone structure enhanced the TAR binding affinities of the unnatural biopolymer.

Specificity of the oligourea-TAR RNA complex formation was addressed by competition experiments (Figure 2c). Oligourea-RNA complex formation was inhibited by the addition of unlabeled wild-type TAR RNA and not by mutant TAR RNAs. Mutant TAR RNA without a trinucleotide bulge (Figure 2c) or with one base bulge (data not shown) was not able to compete for oligourea binding to wild-type TAR RNA.

Two base-pairs immediately above the pyrimidine bulge are critical for Tat recognition.⁹ To determine whether the oligourea recognizes specific base-pairs in the stem region of TAR RNA or only a trinucleotide bulge containing RNA, we synthesized a TAR mutant where the G26-C39 base pair was substituted by a C26-G39 base-pair (Figure 2a). Competition experiments showed that this mutant TAR (G26C) did not inhibit Oligourea binding to TAR RNA (Figure 2c). These results indicate that the tat-derived oligourea can specifically recognize TAR RNA.

To probe the oligourea-RNA interactions and determine the proteolysis stability of oligourea, we synthesized TAR RNA containing 4-thioU at position 23 and performed photocrosslinking experiments as described earlier (Figure 3).^{5, 7} Irradiation of the oligourea-RNA complex yields a new band with electrophoretic mobility less than that of the RNA (lane 4). Both the oligourea and UV (360 nm) irradiation are required for the formation of this crosslinked RNA-oligourea complex (see lanes 3 and 4). Since the crosslinked oligourea-RNA complex is stable to alkaline pH (9.5), high temperature (85 °C) and denaturing conditions (8M urea, 2% SDS), we

-13-

conclude that a covalent bond is formed between TAR RNA and the oligourea during the crosslinking reaction.

To test the protease stability of the oligourea-RNA complexes, we subjected the oligourea-RNA crosslink products to very vigorous proteinase K digestion which showed that the complexes were completely stable and there were no signs of oligo urea degradation (lane 5 and 6). Under similar proteinase K treatment, Tat-TAR photocrosslink products resulted in a complete loss of RNA-protein crosslink and a gain in free RNA as observed by band intensities on the gel.⁵

These findings show that a small tat-derived oligourea binds TAR RNA specifically with high affinity and interacts in the major groove (4-thio groups at U23) of TAR RNA. Due to the difference in backbone structure, oligoureas may differ from peptides in hydrogen-bonding properties, lipophilicity, stability, and conformational flexibility. Moreover, oligoureas are resistant to proteinase K degradation. These characteristics of oligoureas may be useful in improving pharmacokinetic properties relative to peptides. RNA recognition by an oligourea provides a new approach for the design of drugs which will modulate RNA-protein interactions. Transfection enhancing agents could be utilized with drugs comprising the oligourea of the invention to ameliorate any problems associated with the transfection or uptake of the oligourea of the invention.

REFERENCES

1. (a) Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. *Science* 1994, 266, 646-50.
(b) Gottesfeld, J. M.; Neely, L.; Trauger, J. W.; Baird, B. B.; Dervan, P. B. *Nature* 1997, 387, 202-205.
(c) White, S.; Szewczyk, J.; Turner, J.; Baird, E.

-14-

- E.; Dervan, P. B. *Nature* 1998, 391, 468-471.
2. Tinoco, I., Jr.; Puglisi, J. D.; Wyatt, J. R. *Nuci. Acids & Mol. Biol.* 1990, 4, 205-226.
3. Jones, K. A.; Peterlin, B. M. *Annu. Rev. Biochem.* 1994, 63, 717-43.
- 5 4. (a) Puglisi, J. D.; Tan, R.; Calnan, B. J.; Frankel, A. D.; Williamson, J. *Science* 1992, 257, 76-80.
(b) Aboul-ela, F.; Kam, J.; Varani, G. *J Mol. Biol.* 1995, 253, 313-332.
- 10 5. (a) Wang, Z.; Rana, T. M. *J Am. Chem. Soc.* 1995, 117, 5438-5444.
(b) Wang, z.; Rana, T. M. *Biochemistry* 1996, 35, 6491-6499.
(c) Wang, Z.; Wang, x.; Rana, T. M. *J Biol. Chem.* 1996, 271, 16995-16998.
- 15 6. Huq, I.; Rana, T. M. *Biochemistry* 1997, 36, 12592-12599.
7. Wang, X.; Huq, I.; Rana, T. M. *J Am. Chem. Soc.* 1997, 119, 6444-6445.
- 20 8. (a) Cullen, B. R. *Microbiol. Rev.* 1992, 56, 375-394.
(b) Gaynor, R. *AIDS* 1992, 6, 347-363.
(c) Jeang, K.-T.; Berkhout, B.; Dropulic, B. *J Biol. Chem.* 1993, 268, 24940- 24949.
- 25 9. (a) Calnan, B. J.; Biancalana, S.; Hudson, D.; Frankel, A. D. *Genes Dev.* 1991, 5, 201- 210.
(b) Weeks, K. M.; Crothers, D. M. *Cell* 1991, 66, 577-588.
(c) Churcher, M. J.; Lamont, C.; Hamy, F., Dingwall, C.; Green, S. M.; Lowe, A. D.; Butler, P. J. C.; Gait, M. J.; Karn, J. *J Mol. Biol.* 1993, 230, 90-110.
- 30 10. (a) Burgess, K.; Linthicum, D. S.; Shin, H. *Angew. Chem. Int. Ed. Engi.* 1995, 34, 907-909.
(b) Burgess, K.; Ibarzo, J.; Linthicum, D. S.;

-15-

Russell, D. H.; Shin, H.; Shitangkoon, A.; Totani, R.; Zhang, A. J. *J Am. Chem. Soc.* 1997, 119, 1556-1564.

11. Kim, J. M.; Bi, Y. Z.; Paikoff, S. J.; Schultz, P. G. *Tetrahedron Lett.* 1996, 37, 5305- 5308.
- 5
12. Kick, B.; Bllman, J. *J Med. Chem.* 1995, 38, 1427-1430.

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2